VIRUCIDAL HARD-SURFACE EFFICACY TEST PROTOCOL
Feline Calicivirus

For

Envirocleanse LLC
22762 Westheimer Pkwy., Suite 550
Katy, TX 77450

Conducted by

bioX

BioX
8E Industrial Way Salem, NH 03079

Based on Proposal
BIOX-JUN20-17-01

OBJECTIVE:

This test is designed to substantiate virucidal effectiveness claims for a test substance to be labeled as a virucide. It determines the potential of the test substance to disinfect hard surfaces contaminated with Feline Calicivirus (ATCC VR-782) The test is designed to simulate consumer use and conforms to EPA OCSPP 810.2000 and 810.2200 Test substance Performance Test Guidelines, and follows the procedure outlined in the ASTM International test method designated E1053-11, "Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces".

REFERENCES:

- EPA Product Performance Test Guidelines OCSPP 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides Guidance for Efficacy Testing;
- EPA Product Performance Test Guidelines OCSPP 810.2200: Disinfectants for Use on Environmental Surfaces Guidance for Efficacy Testing;
- ASTM International test method designated E1053-11, Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces;
- MB-06-09 Standard Operating Procedure for Germicidal Spray Products as Disinfectants (GSPT): Testing of Staphylococcus aureus, Pseudomonas aeruginosa, and Salmonella enterica
- Series 810 - Product Performance Test Guidelines

TESTING CONDITIONS:

Virus will be dried on a suitable sterile hard surface at ambient temperature. One test substance, two batches (lots), will be tested at one contact time and two replicates (N=2). The test substance will be used to treat the dried virus on a glass Petri dish carrier. After a defined exposure period as specified by the sponsor, the test substance-virus mixture will be scraped from the surface, collected, neutralized and tested for the presence of infectious virions.

MATERIALS:

Test, control and reference substances will be supplied by the sponsor of the study (see last page).

The identity, strength, purity, and composition, or other characteristics which will appropriately define the test, control, or reference substance shall be determined for each batch and shall be documented by the sponsor before its use in a study. Methods of synthesis, fabrication, or derivation of the test, control, or reference substance shall be documented and retained by the sponsor.

When relevant to the conduct of the study the solubility of each test, control, or reference substance shall be determined by the sponsor before the experimental start date. The stability of the test, control, or reference substance shall be determined by the sponsor before the experimental start date or concomitantly according to written standard operating procedures, which provide for periodic analysis of each batch.

The test substance will be tested as supplied by the sponsor unless directed otherwise. All operations performed on the test substance such as dilution or specialized storage conditions must be specified by the sponsor before initiation of testing.

The sponsor assures bioX testing facility management that the test substance has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

bioX will retain all unused test substances for a period of one year upon completion of the test, and then discard them in a manner that meets the approval of the safety officer.

Materials supplied by bioX, including, but not limited to:

- Challenge virus (requested by the sponsor of the study): Calicivirus (ATCC VR-782)
  Note: the virus inoculum will contain 5% serum.
- Host cell line: CrFK cells
- Laboratory equipment and supplies.
- Media and reagents:
  Media and reagents relevant to the virus-host system and test substance being tested will be documented in the first project sheet and data pack.

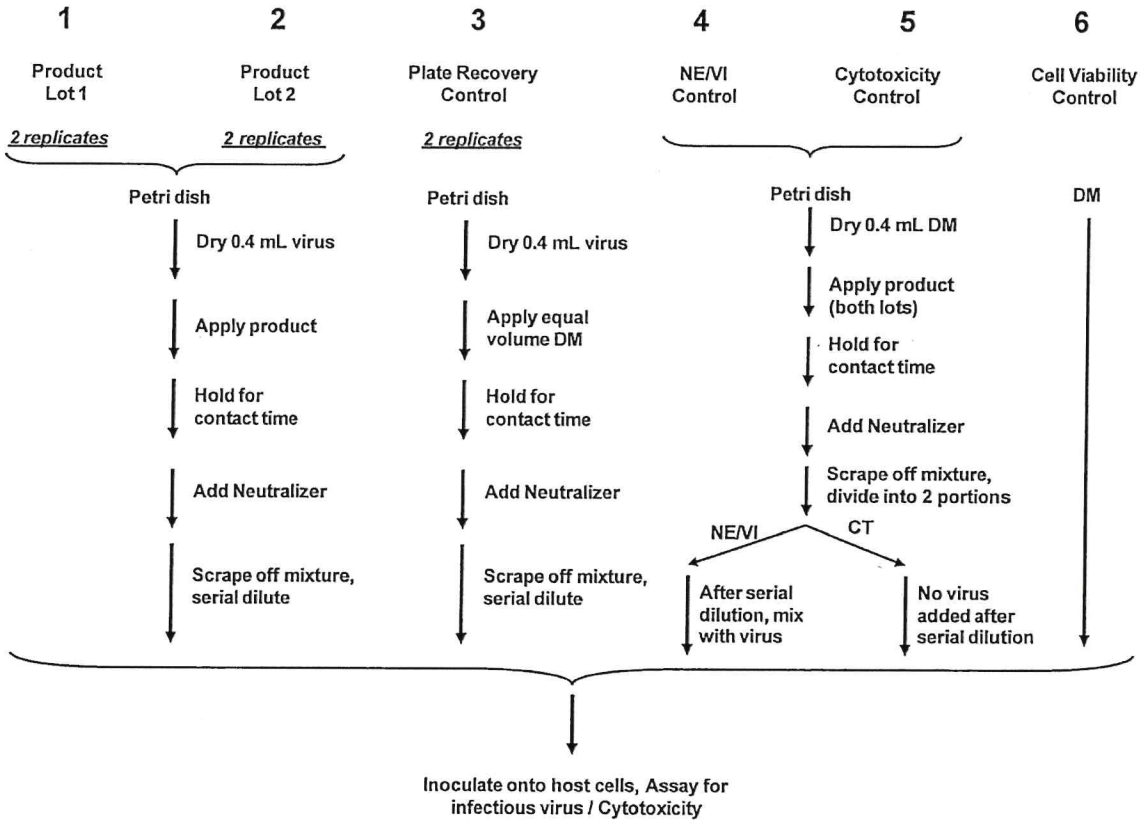
TEST SYSTEM IDENTIFICATION:

All Petri dishes, dilution tube racks, and host-containing apparatus will be appropriately labeled with the following information: virus, host, and test substance and/or project number.

EXPERIMENTAL DESIGN:

This is a non-GLP study. bioX personnel will perform the study per the study flow diagram shown in Figure 1 with steps described in the following sections.

FIGURE 1



DM: Dilution Medium
NE/VI: Neutralizer Effectiveness/Viral Interference control
CT: Cytotoxicity Control

Note: One test substance, two batches (lots), will be tested, at one contact time and two replicates (N=2).
Note 2: The NE/VI and CT will be performed for each lot in singlet runs.

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A. Inoculum preparation:

Viral stocks are purchased from reputable sources that identify them by scientifically accepted methods and may have been propagated at BioX. The virus stocks are stored at an ultra-low temperature.

Frozen viral stocks will be thawed on the day of the test (fresh stock cultures may be used at the discretion of the Study Director). Serum will be added to viral stock to achieve an organic load of 5% (if not already 5%), unless otherwise directed by the Sponsor and pre-agreed by BioX.

Note: a level of approximately 5.0 — 7.0 Log_{10} virus challenge (as indicated by the plate recovery control load), or approximately 3.0 — 5.0 Log_{10} beyond the level of cytotoxicity (if present), should be achieved whenever possible.

B. Carrier preparation:

For each replicate of each lot of the test substance, an aliquot of 0.4 mL of stock virus will be spread over an area of approximately 4 in2 that has been marked on the underside of pre-sterilized glass Petri dishes. Then the virus will be allowed to dry at ambient temperature. The drying time and temperature will be recorded.

Two carriers will be prepared for each lot of test substance using virus. Two carriers will be prepared for the plate recovery control using virus. Additionally, one carrier will be prepared for each lot of test substance for the neutralizer effectiveness/viral interference and cytotoxicity controls using media in lieu of virus as the inoculum.

C. Test substance preparation:

Note: Information on the identity, strength, purity, stability, uniformity, and dose solution analysis of the test substance resides with the sponsor of the study.

The test substance will be prepared according to the sponsor's directions, if provided (see Miscellaneous Information section for details).

No chemical titration of the active ingredient(s) will be performed in this project.

D. Test:

Two lots of the test substance will be tested at one contact time and two replicates (N=2).

For direct liquid application test substance, for each replicate run, after the inoculum has dried, 2.0 mL of the test substance will be added. The dried virus film must be completely covered by the test substance. The plates will remain at the temperature and for the time specified by the sponsor. After the contact period, the test substance will be neutralized with 2.0 mL of appropriate neutralizer and the mixture will be scraped from the surface of the dish with a cell scraper. This will be considered approximately a 10-1 dilution.

For spray type test substance, an aliquot of the test substance, ready-to-use, will be dispensed into a dedicated Electrostatic Sprayer. The test substance reservoir will then be shaken 2-3 times to ensure homogeneity and sprayed to charge the spray bottle. A mock spray action will be performed by applying the test substance as the sponsor directs onto at least two blank Petri dishes. Then the volume dispensed onto each dish will be measured and averaged. This averaged volume from the mock spray runs will be used for the neutralizer for all applicable runs and for the Plate recovery control runs. Then the test substance will be sprayed onto the virus carriers in a horizontal position until thoroughly wet from a distance of 6"-8". Each carrier will be held in a horizontal position for the exposure time as specified by the sponsor. After the contact period, the test substance will be neutralized with an appropriate neutralizer using the averaged volume from the mock spray runs; and the mixture will be scraped off from the surface of the dish with a cell scraper. This post-neutralized sample (PNS) will be considered approximately a 10-1 dilution.

If Sephacryl columns are used to aid in the neutralization and to further reduce the cytotoxicity, each inoculum/test substance/neutralizer mixture sample will be loaded onto a pre-spun Sephacryl column. Following the passage through columns, the eluates will be aseptically collected and serially ten-fold diluted in DM. If columns are not used, serial ten-fold dilutions of the inoculum/test substance/neutralizer mixture will directly be prepared in DM.

E. Infectivity assay:

The residual infectious virus in all test and control samples will be detected by viral-induced cytopathic effect (CPE).

Selected dilutions of the neutralized inoculum/test substance mixture (test samples) and control samples will be added to cultured host cells (at least four wells per dilution, per reaction mixture) and incubated at 36+2°C with 5+1% CO2 for total 7-9 days. The host cells may be washed twice with phosphate buffered saline prior to inoculation. The inoculated culture will be observed and refed with fresh media as necessary, during the incubation period. These activities, if applicable, will be recorded. The host cells will then be examined microscopically for presence of infectious virus. The resulting virus-specific CPE and test substance-specific cytotoxic effects will be scored by examining all test and control samples. These observations will be recorded.

F. Controls:

1. Plate recovery control (PRC):

   This control will be performed in two replicates. The virus inoculum will be spread over the surface of a sterile glass Petri dish and left to dry at ambient temperature. A volume of DM equivalent to that of the test substance will be added to the dried virus. Post-contact time, virus will be subjected to the identical neutralization procedure as the test substance. This control will determine the relative loss in virus infectivity resulting from drying and neutralization alone.

   The results from this control will be compared with the test results to confirm recovery of at least 4.0-Log_{10} of infectious virus in this control following drying and neutralization. Its titer will be used to compare with the titers of the test results to reach the acceptable test criteria (see below).

2. Neutralizer effectiveness/Viral interference control (NE/VI):

   This control will determine if residual active ingredient is present after neutralization and if the neutralized test substance interferes with the virus infection system. This control will be performed for both lots of test substance at one replicate.

   The test substance will be processed exactly as the test procedure but in lieu of virus inoculum, dried DM will be exposed to the test substance and assayed as previously described. Post-treatment and neutralization, the neutralized DM/test substance mixture will be divided into two portions, one for cytotoxicity control and the other for neutralizer effectiveness/viral interference control, and processed as the test.

   If columns are used, each portion will be passed through individual columns and the eluate will be serially diluted ten-fold in DM. If columns are not used, each portion will be directly diluted using serial ten-fold dilutions in DM.

   The neutralizer effectiveness/viral interference control sample will be diluted as

follows: using dilution test tubes and appropriate pipette, an aliquot of the PNS will be used for making serial 10-fold dilutions in DM (for example, 0.5 mL sample + 4.5 mL DM). Following serial dilution, 0.1 mL of a low titered virus, containing approximately 1,000 — 5,000 infectious units of virus, will be added to 4.5 mL of each dilution and held for a period of no shorter than the contact time. Then these samples will be used to inoculate host cells as described for the test procedure.

Selected dilutions of the sample will be added to cultured cell monolayers at a minimum of four wells per dilution per sample, as described in Section F, "Infectivity Assay".

3. Cytotoxicity control (CT):

   This control will be performed for both lots of test substance at one replicate.

   The cytotoxicity sample, acquired from the neutralizer effectiveness/viral interference control run, will be diluted and have no virus added. Selected dilutions will be inoculated and incubated in the same manner as the rest of the test and control samples. These effects are distinct from virus-induced cytopathic effects, which will be evident in the plate recovery control cultures.

4. Column titer control (to be performed only if a Sephacryl column is used):

   This control will be performed to determine any affect the columns may have on infectious virus titer. It will be performed in two replicates.

   The sample for this control will be acquired from a portion of the PRC, prior to passing through the columns and will be serially diluted in DM, then processed in the same manner as the test.

5. Cell viability control:

   This control will be performed in a single run. It will demonstrate that cells remain viable throughout the course of the assay period. In addition, it will confirm the sterility of the DM employed throughout the assay period. At least four wells of cells will receive only DM and will be incubated and processed with both test and other controls. This will serve as the negative control.

6. Virus Stock Titer control (VST)

   This control will be performed in a single run. An aliquot of the virus used in the study will be directly serially diluted and inoculated onto the host cells to confirm the titer of the stock virus. This control will demonstrate that the titer of the stock virus is appropriate for use and that the viral infectivity assay is performed appropriately.

G. Calculation:

The 50% tissue culture infective dose per mL ($TCID_{50}$/mL) will be determined using the method of Spearman-Karber (Karber G., Arch. Exp. Pathol. Pharmakol. 1931, 162: 480-483) or other appropriate methods such as Reed and Muench (Am. J. of Hyg. 1938,

27:493). In the case where a sample contains no detectable virus, a statistical analysis may be performed based on Poisson distribution (International Conference on Harmonization, Topic QSA, 1999: 24-25) to determine the theoretical maximum possible titer for that sample. These analyses will be described in detail in the final report. The test results will be reported as reduction of the virus titer post treatment with the test article expressed as Log_{10}.

The Virus Load will be calculated in the following manner:
Virus Load (Log_{10} on $TCID_{50}$) = Virus Titer (Log_{10} $TCID_{50}$/mL) + Log_{10} [Volume per sample (mL)]

The Log_{10} Reduction Factor (LRF) will be calculated in the following manner:
Log_{10} Reduction Factor = Initial viral load (Log_{10} $TCID_{50}$) — Output viral load (Log_{10} $TCID_{50}$)

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance.

- The infectious virus recovered from the PRC control must be ≥4.0-Log_{10}
- $TCID_{50}$ units.
- Viral-induced cytopathic effect must be distinguishable from test substance induced cytotoxic effects (if any).
- Virus must be recovered from the neutralizer effectiveness/viral interference control (not exhibiting cytotoxicity).
- The Cell Viability Control (assay negative control) must not exhibit virus.

TEST SUBSTANCE EVALUATION CRITERIA:

According to the US Environmental Protection Agency, the test substance passes the test if there is complete inactivation of the virus at all dilutions. When cytotoxicity is evident, at least a three-log reduction in titer must be demonstrated beyond the cytotoxic level.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned prior to initiation of the test. Resumes are maintained and are available on request. This study will be conducted at bioX, 8E Industrial Way, Salem, NH 03079.

REGULATORY COMPLIANCE AND QUALITY ASSURANCE (GLP studies only):

This is a non-GLP study and therefore the GLP requirements under 40 CFR 160 are not included in the test procedure.

PROTOCOL AMENDMENTS AND DEVIATIONS:

Any protocol amendment(s) and protocol deviation(s) identified will be reported in project sheet(s)

and included in the final report.

REPORT FORMAT:

bioX employs a standard report format for each test design. Each final report will provide at least the following information:

- Sponsor identification
- Test substance identification
- Type of assay and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results presented in tabular form
- Methods and evaluation criteria, if applicable
- Dates of study initiation and completion (GLP studies only)
- Signed Quality Assurance and Compliance Statements (GLP studies only)
- Certificate of Analysis (for GLP studies only; if provided by the Sponsor)

RECORDS TO BE MAINTAINED:

For all GLP studies, the original signed final report will be sent to the Sponsor.

All raw data, protocol, protocol modifications, test substance records, and copy of final report will be stored in archives at bioX, 8E Industrial Way, Salem, NH 03079, virtually, or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

Additional information about the test substance; challenge virus and host cell line monolayers used and the type of neutralizers employed in the test will be addressed in a project sheet issued separately for each study. All project sheets issued will be forwarded to the study sponsor for appropriate action.

MISCELLANEOUS INFORMATION:

The following information is to be completed by sponsor prior to the initiation of study:

A. Name and address: Envirocleanse LLC
22762 Westheimer Pkwy., Suite 550
Katy, TX 77450

B. Test substance information:

Test substance name	Envirocleanse A					
Active ingredient(s)	Hypochlorous Acid					
Lot No.	Lot 1			Lot 2		
	Batch #4			Batch #2		
Manufacture Date	01Jul20			01Jul20		
Lower Certified Limit (LCL)	Yes	No	**X**N/A	Yes	No	**X**N/A
Dilution	**X** Ready-to-use; or ____ (1 part concentrate + ____parts diluent)					
Diluent	**X** Not applicable (Ready-to-use); or ____ppm (+ 2.9%) AOAC hard water					
MSDS Certificate Of Analysis	**X** Provided Not provided					

Test Conditions:

Contact time	**X**10 minutes
Contact temperature	Ambient Room Temperature (20+1C)
Spray application	Not applicable (i.e., direct soaking); or **X** Spray from 6-8 inches until thoroughly wet
Organic load	5% serum in viral inoculum

MISCELLANEOUS INFORMATION (Continued):

REPORT HANDLING:

The sponsor intends to submit this information to: X US EPA

STUDY CONDUCT: X Non-GLP
GLP

PROTOCOL APPROVAL:

Sponsor Signature: _____ Date: 9/3/20

Printed Name: MATTHEW T. DENNIS
ENVIROCLEANSE, LLC

Study Director Signature: _____ Date: 09/03/2020

Study Director Printed Name: Mark McElligott

bioX, 8E Industrial Way, Salem, NH 03079

Date Issued: 03Sep20 Project Sheet No. 1 Page No. 1 Laboratory Project Identification No. BIOX-EC-JUN20-17-01			
STUDY TITLE: VIRUCIDAL HARD-SURFACE EFFICACY TEST- Feline Calicivirus	STUDY DIRECTOR: Mark McElligott 09/03/2020 Signature Date		
TEST MATERIALS (Product) Envirocleanse A	LOT NO. Batch #2 and Batch #4	DATE RECEIVED: 01Jul20	DS NO N/A
	STORAGE CONDITIONS: Cabinet Storage in Ambient Temperature Controlled Room		
PROTECTIVE PRECAUTION REQUIRED: See MSDS			
PHYSICAL DESCRIPTION: Liquid in Opaque Gallon Containers			
PURPOSE: See attached protocol. AUTHORIZATION: See client signature.			
CONDUCT OF STUDY: EPA, non-GLP			

SPONSOR: Envirocleanse, LLC 22762 Westheimer Pkwy., Suite 550 Katy, TX 77450	CONTACT PERSON: Matthew Dennis

TEST CONDITIONS:

Challenge organism: Feline Calicivirus (FCV), Strain: F9, ATCC VR-782

Host cell line: CrFK cells, ATCC CCL-94

Active ingredient(s): Hypochlorous acid

Organic load: 5% serum in viral inoculum

Dilution medium: RPMI 1640 + 2% Newborn Calf Serum (NCS)

Neutralizer: RPM1 1640 + 10% NCS + 0.5% Na2S O3

Dilution: Ready to use

Contact time(s): 10 minutes

Contact temperature: Ambient Room Temperature (20+1°C)

Incubation time: 7 — 9 days

Incubation temperature: 36+2°C with 5+1% CO

Comments: Spray Application: Spray from 6" — 8" until thoroughly wet